Developmental Biology ■ (■■■■) ■■■–■■■

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

DB Letters

Vegfa signaling promotes zebrafish intestinal vasculature development through endothelial cell migration from the posterior cardinal vein

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ARTICLE INFO

Article history:
Received 16 June 2015
Received in revised form
12 December 2015
Accepted 4 January 2016

Keywords:
Zebrafish
Vegf
Intestinal
Vascular endothelial
Organ

ABSTRACT

The mechanisms underlying organ vascularization are not well understood. The zebrafish intestinal vasculature forms early, is easily imaged using transgenic lines and in-situ hybridization, and develops in a stereotypical pattern thus making it an excellent model for investigating mechanisms of organ specific vascularization. Here, we demonstrate that the sub-intestinal vein (SIV) and supra-intestinal artery (SIA) form by a novel mechanism from angioblasts that migrate out of the posterior cardinal vein and coalesce to form the intestinal vasculature in an anterior to posterior wave with the SIA forming after the SIV. We show that *vascular endothelial growth factor aa (vegfaa)* is expressed in the endoderm at the site where intestinal vessels form and therefore likely provides a guidance signal. Vegfa/Vegfr2 signaling is required for early intestinal vasculature development with mutation in *vegfaa* or loss of Vegfr2 homologs causing nearly complete inhibition of the formation of the intestinal vasculature. Vegfc and Vegfr3 function, however, are dispensable for intestinal vascularization. Interestingly, ubiquitous overexpression of Vegfc resulted in an overgrowth of the SIV, suggesting that Vegfc is sufficient to induce SIV development. These results argue that Vegfa signaling directs endothelial cells to migrate out of existing vasculature and coalesce to form the intestinal vessels. It is likely that a similar mechanism is utilized during vascularization of other organs.

1. Introduction

Proper vascularization is necessary for development of the embryo and survival of the adult. A functional vascular network is required for the transport of nutrients, hormones, immune cells, and oxygen to cells, as well as the removal of potentially toxic metabolic waste products. The early development of major blood vessels, as well as the signals that promote vascular development, has been well characterized; however, little is known about organ-specific vascularization. Two distinct mechanisms have been implicated in vascular development. Vasculogenesis involves the differentiation of angioblasts from mesoderm and the formation of primitive blood vessels from angioblasts at the site of their origin, while angiogenesis refers to the formation of new vasculature from preexisting vessels by sprouting or vessel remodeling (Sabin, 1917). It has been suggested that organ specific vasculature develops by a combination of vasculogenesis and angiogenesis. For example, in the human liver, arteries are thought to develop by angiogenesis while highly fenestrated capillaries have been proposed to form by vasculogenesis from intra-hepatic mesenchyme (Gouysse et al., 2002). During mouse liver development, loose endothelial cells have been observed that intercede between the hepatic endoderm cells and the septum transversum mesenchyme (Kingsbury et al., 1956; Matsumoto et al., 2001; Sherer, 1991). However, the source of these endothelial progenitors has not been known and the genetic pathways involved in the different mechanisms of organ vascularization have not been studied in detail in any model system. Understanding the mechanism that leads to organ vascularization can give insight into the formation of vascular malformations and pathological angiogenesis.

The zebrafish has become an exceptional model for studying vascular development due to the transparency of the embryo and the ability for genetic manipulations (Bradbury, 2004). As in other vertebrate organisms, the cardiovascular system is among the first to form, with circulation beginning by approximately 24 h post

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http://dx.doi.org/10.1016/j.ydbio.2016.01.002
0012-1606/

fertilization (hpf) (Fishman and Chien, 1997). The mechanisms and pathways involved in vascular development have also been shown to be highly conserved among vertebrates (Jin et al., 2005; Pardanaud et al., 1987). The axial vessels—the dorsal aorta (DA) and posterior cardinal vein (PCV)—develop during mid-somitogenesis from progenitors that originate bilaterally in the lateral plate mesoderm and migrate to the midline in two distinct waves, where they coalesce (Kohli et al., 2013; Torres-Vazquez et al., 2003; Zhong, 2005). Following formation of the axial vessels, intersomitic arteries sprout from the DA driven by Vegfa-Vegfr2 signaling (Bahary et al., 2007; Habeck et al., 2002; Siekmann and Lawson, 2007) and intersomitic veins from the PCV driven by Vegfc-Vegfr3 signaling (Hogan et al., 2009a), both at the somite boundaries.

Vascular endothelial growth factor (Vegf) signaling is required for development of blood vessels by directing the migration and survival of endothelial cells. This requirement of Vegf signaling for functional vascular development has been demonstrated in mouse (Carmeliet et al., 1996; Ferrara et al., 1996), *Xenopus* and chick (Cleaver and Krieg, 1998), as well as zebrafish (Lawson et al., 2002; Nasevicius et al., 2000). As in other vertebrates, zebrafish have multiple Vegf ligand and receptor pathways including Vegfa-Vegfr2 and Vegfc-Vegfr3. Zebrafish express two VEGFR2 homologs known as Kdrl/Flk1 and Kdr/Kdrb, and a single VEGFR3 homolog Vegfr3/Flt4 (Covassin et al., 2006). It has also been suggested that Kdrl could represent Vegfr4, a Vegf receptor that has been lost in mammals (Bussmann et al., 2008). Morpholino knockdown of *vegfaa,* one of the two mammalian Vegfa homologs in zebrafish, results in a loss of intersomitic vessels (ISVs) and caudal plexus, in addition to failure of axial vessel patterning, and lack of circulation (Childs et al., 2002; Nasevicius et al., 2000), while the combined knockdown of *VEGFR2* homologs by injecting *kdr* morpholino (MO) into *kdrl* mutant embryos exhibits a milder yet similar phenotype (Covassin et al., 2006). Knockdown of Vegfc-Vegfr3 signaling results in loss of lymphatic vessels, as well as the inhibition of venous intersomitic vessel sprouting (Covassin et al., 2006; Karkkainen et al., 2004; Siekmann and Lawson, 2007).

The intestinal vasculature in zebrafish is comprised of the supra-intestinal artery (SIA) and sub-intestinal vein (SIV) which are among the next vessels to develop in the trunk, following the axial vessels and ISVs. These vessels develop close to the surface in a highly stereotypic pattern and therefore are an excellent model to study organ specific vascularization. As analyzed by microangiography, the intestinal vasculature begins developing approximately 2.5 dpf and is complete by 4 dpf. The SIV begins to develop at 2.5 dpf as two bilateral vessels, of which the right vessel further develops, while the left vessel breaks up to form hepatic vasculature and loops to the right SIV by 4 dpf. The SIA develops around 3 dpf as an extension of the anterior mesenteric artery, and later forms connections with the SIV (Isogai et al., 2001). While the structure and formation of these vessels has been characterized, the mechanism driving the formation and the lineage of the endothelial cells that make up the vessels have not been identified.

Various studies have noted effects on the development of the SIV following genetic manipulation; however, an in detail examination has not been conducted. Vegf inhibition by chemical inhibitor and *vegfaa* morpholino results in a failure of SIV formation, and overexpression of *vegfaa165* results in expansion of the SIV (Hao et al., 2010; Kawamura et al., 2008). In addition, overexpression of *bmp2b* or loss of microsomal triglyceride transfer protein results in ectopic sprouting from the ventral region of the SIV (Avraham-Davidi et al., 2012; Wiley et al., 2011). Additionally, MO knockdown of *survivin* or *vegfab* result in loss of SIV development (Bahary et al., 2007; Ma et al., 2007).

In this study, we demonstrate that the intestinal vasculature in zebrafish does not form by vasculogenesis *de novo* as it has been suggested by earlier studies in mammalian models, but rather that it forms via the migration of individual endothelial cells derived from the posterior cardinal vein which coalesce to form the sub-intestinal vein and the supra-intestinal artery. This is a novel mechanism, which differs from standard definitions of vasculogenesis and angiogenesis. We also examine the roles of multiple Vegf ligands and receptors and identify, through morphant and mutant analysis, Vegfaa-Vegfr2 signaling as essential for proper patterning of the intestinal vasculature. Interestingly, despite its role in venous ISV development, Vegfc-Vegfr3 signaling appears to be dispensable for the intestinal vasculature formation based on mutant analysis. However, we demonstrate that overexpression of *vegfc* results in ectopic ventral sprouts from the SIV, suggesting that Vegfc signaling is sufficient to induce SIV sprouting. Together these results demonstrate a working model of organ specific vascularization in which terminally differentiated endothelial cells migrate from existing vasculature to coalesce in forming new vessels. Because the molecular mechanisms in vascular development are highly evolutionarily conserved, it is likely that organ-specific vascularization also proceeds similarly in other vertebrates.

2. Materials and methods

2.1. Zebrafish lines and embryos

The following zebrafish lines were used in experiments: Tg(*fli1a:*EGFP)^{y1} (Lawson and Weinstein, 2002), Tg(*kdrl:*mCherry)^{ci5} (Proulx et al., 2010), Tg(*etv2:*mCherry)^{zf373} (Veldman and Lin, 2012), Tg(*fli1a:*nEGFP)^{y7} (Roman et al., 2002), TgBAC(*etv2:*Kaede)^{ci6} (Kohli et al., 2013), Tg(*tp1:eGFP*)^{um14} (Parsons et al., 2009), *vegfc*^{um18} (Villefranc et al., 2013), Tg(*hsp70:*Vegfc)^{ci25} (Davis et al., unpublished), *flt4*^{sa9798} (Busch-Nentwich et al., 2013), *flt4*^{hu4602} (Hogan et al., 2009b), *vegfaa*^{bn1}, and *kdrl*^{um19} (Covassin et al., 2009). Embryos were incubated at 28.5 °C and staged using criteria previously described (Kimmel et al., 1995). Embryos beyond 24 h post fertilization (hpf) were treated with 1-phenyl-2-thiourea (PTU) to inhibit the formation of pigment. *kdrl*$^{-/-}$ embryos were identified at 24–48 hpf by failure of inter-segmental vessel extension and *vegfaa*$^{-/-}$ embryos were identified by failure to develop normal axial and intersomitic vasculature.

2.2. In situ hybridization

In situ hybridization was performed as previously described (Jowett, 1999). Anti-sense riboprobes for *kdrl, fli1a, vegfc* (Cermenati et al., 2013), *kdr* (Covassin et al., 2006), and *flt4* labeled with DIG-UTP were synthesized using T7 RNA polymerase (Ambion/Promega) as previously described (Thompson et al., 1998). *vegfaa* probe was synthesized from PCR products derived from pCS2-*vegfaa*$_{121}$ plasmid (Liang et al., 2001) using the primers below designed with an integrated T7 promoter site. Processed embryos were dehydrated in 100% ethanol for storage at −20 °C to improve contrast then rehydrated in PBS and mounted in 3% methylcellulose under cover slips for imaging. An AxioImager compound microscope with Plan-Neofluar 10X/0.3 NA objective and AxioCam ICC3 color camera (Carl Zeiss Inc., USA) controlled using AxioVision 4.6 software (Carl Zeiss Inc., USA) was used to capture Z-stack images and produce extended focus images. Image contrast and brightness was adjusted using Adobe Photoshop CS6.

Primer sequences: *vegfaa* probe fw:

5′-TTATTTCTCGCGGCTCTCCTC-3′, *vegfaa* probe rev:

5′-GAAATTAATACGACTCACTATAGGGCATCTTGGCTTTTCA-CATCTTTCT-3′.

Please cite this article as: Koenig, A.L., et al., Vegfa signaling promotes zebrafish intestinal vasculature development through endothelial cell migration from the posterior cardinal vein. Dev. Biol. (2016), http://dx.doi.org/10.1016/j.ydbio.2016.01.002